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DEPARTMENT OF THE ARMY
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EFFECTS OF BASIC PROTEINS AND POLYAMINO ACIDS ON INFECTIOUS RNA OF EQUINE ENCEPHALITIS VIRUSES

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ABSTRACT

Methylated bovine serum albumin has previously ·en shown to "mask" reversibly the infe^ctivity of infectious ribonucleic acid (IRNA) from Venezuelan equine encephalitis (VEE) virus and to protect the nucleic acid against inactivation by micrococcal nuclease. In studies reported here, the effects of several other basic proteins and synthetic polyamino acids on the infectivity of VEE-IRNA and eastern equine encephalitis virus IRNA have been compared. Quantitative differences were observe' among the proteins and the polyamino acids in their capacity to .ower infectivity. Polylysine and polyornithine completely masked .nfectivity, as measured by plaque formation, when present in on'y 0.01 to 0.001 times the concentration required for proteins to be effective. The original infectivity of the IRNA before treatment was recovered when the polylysine-IRNA complex was treated with pronase, indicating that the presence of the polyamino acid did not degrade the infectious unit. Protection of IRNA against inactivation by pancreatic ribonucleasr was also demonst-ated in the presence of polylysine, providing further evidence tnat a complex was formed between the nucleic acid and the polyamino acid.

EFFECTS OF BASIC PROTEINS AND POLYAMINO ACIDS ON INFECTIOUS RNA OF EQUINE ENCEPHALITIS VIRUSES

The adsorption of ribonucleic acids (RNA) on columns of methylated bovine serum albumin (MBSA) - kieseiguhr was shown by Mandell and Hershey[1] to be dependent on salt concentration. Plescia et al.[2] showed that MBSA forms complexes with many species of nucleic acids. Recently, Norrell and Costlow[3] reported that the infectious RNA (IRNA) of Venezuelan equine encephalitis (VEE) virus formed a noninfectious complex with 0.1% MBSA at low concentrations of NaCl and that complex formation and loss of infectivity were reversed when the salt concentration was increased to 1 M. In related studies in our laboratory, we examined the effect of pancreatic ribonuclease on the MBSA-IRNA complex and investigated the comparative abilities of other basic proteins and some synthetic polyamino acids to form complexes with IRNA.

The IRNA preparations used here were extracted with hot phenol and sodium dode . sulfate from VEE or eastern equine encephalitis (EEE) virus that was partially purified from supernatant growth medium of infected chick fibroblast (CF) monolayer cultures. The preparations had specific infectivities of approximately 1 x 10^5 plaque-forming units (pfu) per µg of RNA. RNA was determined quantitatively by measuring the optical density of concentrated samples at 260 mµ. Infectivity was assayed by plaque formation in monolayer cultures of CF cells that were pretreated with hypertonic NaCl.[4]

Table 1 shows data from a typical study of the effect of pancreatic ribonuclease on a complex of VEE-IRNA and MBSA. Final concentrations of the various components in the incubation mixtures were 1 mg MBSA/ml, approximately 0.5 µg IRNA/ml, and 0.01 µg ribonuclease/ml. Incubation was at 4 C in phosphate buffer, and samples were diluted 100-fold in 1 M NaCl immediately following incubation to stop ribonuclease activity and to dissociate the MBSA-IRNA complex. When diluted in isotonic NaCl, infectivity of the IRNA in the presence of MBSA was less than 1 x $10^{2.7}$ pfu/ml. Although the enzyme treatment inactivated 99.9% of the control IRNA without MBSA, there was no significant loss of infectivity of the IRNA that had been complexed with MBSA during the enzyme treatment.

The very high proportion of MBSA to RNA required to obtain complete masking of infectivity was a disadvantage in some of the studies planned. Therefore, we examined some other basic proteins and a synthetic polyamino acid, polylysine, for their capability to form reversible complexes with IRNA. Table 2 shows results from a typical study with a number of proteins each at a concentration of 100 µg/ml. The proteins were mixed with aliquots of a sample of VEE-IRNA in isotonic NaCl at room temperature. Duplicate sets of dilutions were then prepared in 0.15 M and 1.0 M buffered NaCl

TABLE 1. PROTECTION OF METHYLATED BOVINE SERUM ALBUMIN OF VEE-IRNA AGAINST RIBONUCLEASE

Composition of Sample	Ribonuclease[a]	Titer, pfu/ml
IRNA	None	1 x $10^{6.1}$
IRNA + MBSA (1 mg/ml)	None	1 x $10^{6.1}$
IRNA	0.01 μg/ml	<1 x $10^{3.0}$
IRNA + MBSA (1 mg/ml)	0.01 μg/ml	1 x $10^{6.0}$

a. Ribonuclease was Worthington pancreatic (3X crystallized). All samples were incubated for 5 minutes at 4 C. Samples were diluted 100-fold in 1 M NaCl immediately following enzyme treatment to stop ribonuclease activity.

for assay on CF monolayers that had been pretreated with hypertonic NaCl. As seen here, methylated bovine gamma globulin (MBGG), two different lots of histone from calf thymus, and polylysine at 100 μg/ml were more effective than MBSA in masking infectivity of the IRNA. However, in the presence of these more effective compounds, the original infectivity of the IRNA was not recovered by raising the salt concentration. Thus, it was not clear from these data whether the loss of infectivity was caused by denaturation of the IRNA or by failure to dissociate a complex. We chose to confine our efforts to studies with histone and polylysine, which were the most effective substances in masking infectivity at a concentration of 100 μg/ml.

TABLE 2. COMPARISON OF EFFECT OF BASIC PROTEINS IN REVERSIBLE MASKING OF INFECTIVITY OF VEE-IRNA

Protein, 100 μg/ml	Titer of IRNA, pfu/ml[a]	
	Diluted in 0.15 M NaCl	Diluted in 1 M NaCl
None	1 x $10^{5.7}$	1 x $10^{5.8}$
Lysozyme	1 x $10^{5.8}$	1 x $10^{5.9}$
Methylated albumin	1 x $10^{5.1}$	1 x $10^{5.5}$
Methylated gamma globulin	1 x $10^{4.1}$	1 x $10^{4.8}$
Histone A[b]	<1 x $10^{2.7}$	1 x $10^{3.7}$
Histone B[b]	<1 x $10^{2.7}$	1 x $10^{3.3}$
Polylysine	<1 x $10^{2.7}$	<1 x $10^{2.7}$

a. All samples were assayed on monolayer cultures of CF cells that had been pretreated with hypertonic NaCl.
b. Histone A and B were two different lots of histone from calf thymus.

Preliminary determinations were made with these substances to ascertain the minimal concentrations required to effect significant loss of infectivity. Figure 1 shows data obtained when a sample of EEE-IRNA, at a concentration of approximately 1 μg/ml and 1 x $10^{4.7}$ pfu/ml, was incubated for 5 minutes at room temperature with histone at the indicated concentrations. Samples were incubated in isotonic NaCl and then assayed from the isotonic diluent. A quantitative relationship can be observed between histone concentration and loss of infectivity. Also, 90% of the infectivity was lost when histone was present in a concentration 10 times that of the IRNA. Similar results were obtained in experiments with histone and VEE-IRNA.

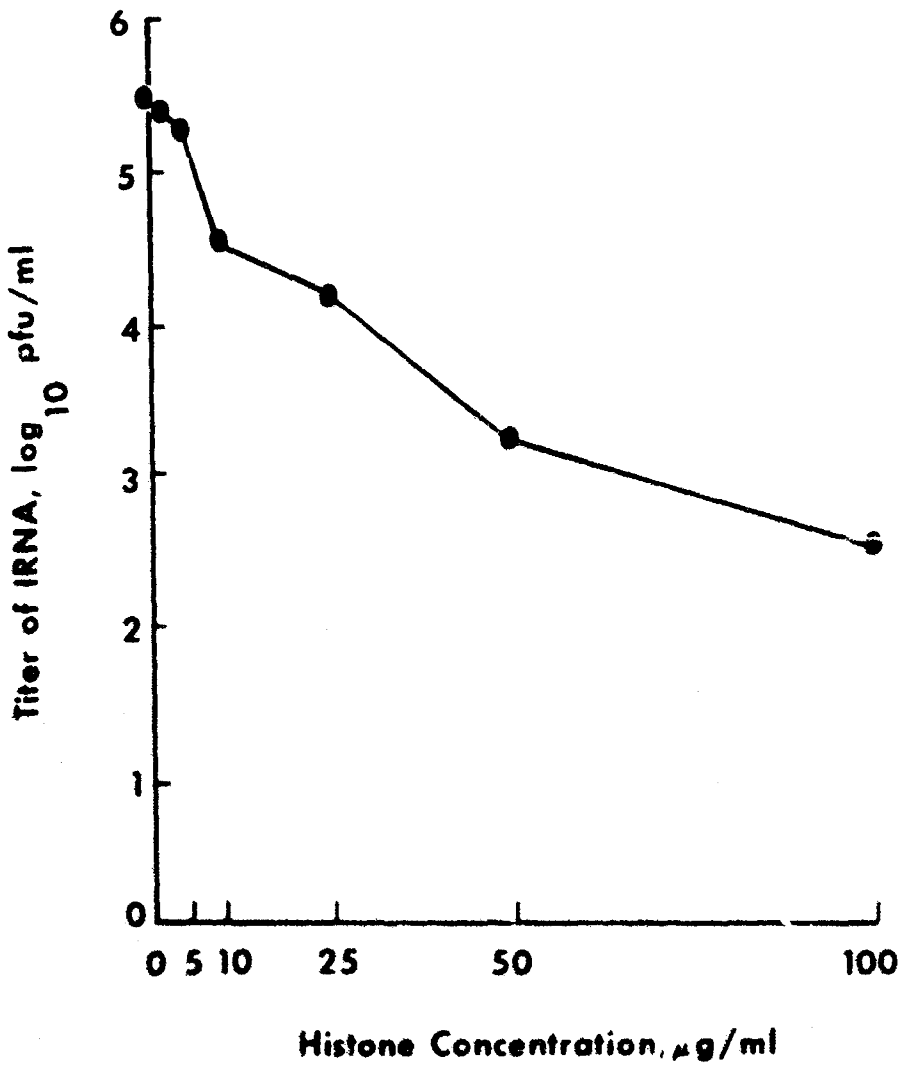

Figure 1. Effect of Histone Concentration on Inactivation of EEE-IRNA.

Preliminary studies with polylysine that had a molecular weight range of 100,000 to 200,000 showed that this polyamino acid, when present in only five to 10 times the concentration by weight of VEE- or EEE-IRNA, was capable of masking more than 99.9% of the infectivity. Results from a study with VEE-IRNA shown in Table 3 indicated even greater efficiency in effecting loss of infectivity. When polylysine and IRNA were present in approximately equal concentrations by weight, only 0.1% of the original infectivity was detected. Furthermore, when the polylysine-IRNA preparation containing 1 µg each of polylysine/ml and IRNA/ml was treated for 45 minutes at 37 C with 10 units of pronase/ml to digest the polyamino acid, the original infectivity was completely recovered.

TABLE 3. REVERSIBLE MASKING OF INFECTIVITY OF VEE-IRNA BY POLYLYSINE

Polylysine Concn, µg/ml	Ratio (w:w) Polylysine:IRNA	Titer of IRNA,a/ pfu/ml
Untreated		
2	2:1	$<1 \times 10^{2.7}$
1	1:1	$1 \times 10^{2.7}$
0.2	1:5	$1 \times 10^{5.0}$
0.1	1:10	$1 \times 10^{5.8}$
None	-	$1 \times 10^{5.8}$
After pronase treatmentb/		
1	1:1	$1 \times 10^{5.6}$
None	-	$1 \times 10^{5.6}$

a. Samples were diluted in buffered 0.15 M NaCl and assayed on monolayers of CF cells that had been pretreated with hypertonic NaCl.
b. Samples were treated with 10 units of pronase/ml for 45 minutes at 37 C.

It was of interest to determine whether polylysine protected IRNA against inactivation by ribonuclease in addition to masking infectivity. Table 4 shows data from a typical experiment with VEE-IRNA. In this study, polylysine was present in approximately two times the concentration by weight of the IRNA. Samples of IRNA in the presence and absence of polylysine were treated with 0.001 µg of pancreatic ribonuclease/ml for 5 minutes at 37 C, then diluted and treated for 30 minutes at 37 C with 2.5 units of pronase/ml to digest the polylysine. More than 10 times as much infectivity remained in the polylysine-IRNA as in the IRNA alone after the ribonuclease treatment. The pronase treatment used in this experiment was not adequate to effect complete reversal of the polylysine effect. Therefore, the protection against ribonuclease may be more effective than indicated by these preliminary results.

TABLE 4. PROTECTION BY POLYLYSINE OF VEE-IRNA AGAINST INACTIVATION BY PANCREATIC RIBONUCLEASE

Polylysine Concn, µg/ml	Treatment		Titer of IRNA, pfu/ml
	Ribonuclease, 5 min, 37 C[a]	Pron se, 30 min, 37 C	
None	None	None	$1 \times 10^{5.8}$
None	0.001 µg/ml	2.5 units/ml	$1 \times 10^{3.8}$
2.5	0.001 µg/ml	2.5 units/ml	$1 \times 10^{5.0}$
2.0	None	2.5 units/ml	$1 \times 10^{5.1}$

a. Immediately following first treatment, samples were diluted 20-fold to stop ribonuclease activity.

Results reported here indicate that polylysine does complex with IRNA without irreversibly altering the integrity of the nucleic acid. Polylysine was more efficient in masking infectivity than any of the basic proteins studied. When the ratio of lysine units in the polyamino acid to nucleotide units in the IRNA was as low as two to one, only 0.1% of the original infectivity was detected. No quantitative difference in capacity to form complexes with polylysine was observed between IRNA from VEE or EEE virus.

In preliminary studies to compare the effects of polylysine with those of polyornithine, the latter was quantitatively less effective in masking the infectivity of VEE-IRNA. Because the isoelectric points of lysine and ornithine are very similar, it appears that basicity was not a factor in the difference between the effectiveness of the two polyamino acids. The difference between the two in their capacity to form complexes with IRNA may be influenced by one or both of the following known differences: (i) the chain length of the polyornithine was one-fourth to one-half that of the polylysine used, and (ii) the ornithine unit is shorter by one carbon atom than the lysine unit. Sober et al.[5] showed that polylysine and RNA formed complexes containing equivalent amounts of polyamino acid and RNA on a charge basis. It is possible that the shorter length of the units in polyornithine prevents it from combining with RNA as efficiently as polylysine.

Further, more detailed examination of the complexes considered here and the differences among them may help to characterize the viral nucleic acid with respect to its infectivity.

Experiments are being conducted to compare the effects of polylysine and polyornithine on IRNA. Physical studies on the polyamino acid - IRNA complexes are also under way.

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Methylated bovine serum albumin has previously been shown to "mask" reversibly the infectivity of infectious ribonucleic acid (IRNA) from Venezuelan equine encephalitis (VEE) virus and to protect the nucleic acid against inactivation by micrococcal nuclease. In studies reported here, the effects of several other basic proteins and synthetic polyamino acids on the infectivity of VEE-IRNA and eastern equine encephalitis virus IRNA have been compared. Quantitative differences were observed among the proteins and the polyamino acids in their capacity to lower infectivity. Polylysine and polyornithine completely masked infectivity, as measured by plaque formation, when present in only 0.01 to 0.001 times the concentration required for proteins to be effective. The original infectivity of the IRNA before treatment was recovered when the polylysine-IRNA complex was treated with pronase, indicating that the presence of the polyamino acid did not degrade the infectious unit. Protection of IRNA against inactivation by pancreatic ribonuclease was also demonstrated in the presence of polylysine, providing further evidence that a complex was formed between the nucleic acid and the polyamino acid.

14. Key Words
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